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# A new cost-effective, selective and differential medium for the isolation of *Cronobacter* spp.

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ABSTRACT

The objective of the present study was to develop a new selective, differential and cost-effective medium (Kim and Rhee — KR-medium) for the isolation of *Cronobacter spp*. In this new medium, which contained salicin as a differential agent, *Cronobacter* spp. generated typical colonies with characteristic violet-colored centers surrounded by a transparent to opalescent border, and the growth of other microorganisms (40 strains) was inhibited or produced visually distinguishable colonies. Using healthy and heat- and desiccation-injured cells, the quantity of nutrients was adjusted to determine the optimal recovery rate, selectivity, differentiation and cost-effectiveness. Peptone and salicin concentrations were established as 10 and 8 g/L, respectively. The KR medium was then validated using salicin fermenting organisms, including *Cronobacter* spp. (52 strains), *Enterobacter cloacae* (50 strains) and *Klebsiella pneumonia* (10 strains) isolated from clinical and food specimens. All strains of *Cronobacter* spp. produced typical colonies and other salicin fermenting organisms were easily distinguishable from *Cronobacter* spp. with the exception of 2 *E. cloacae* strains. The verification of KR medium was carried out in powdered infant formula artificially inoculated with healthy, heat-injured, and desiccation-injured *Cronobacter* spp. and the expected typical colonies were appeared. The KR medium had a high specificity (98%) and sensitivity (100%), with no false-negative results. Moreover, we show that the cost of the KR medium is much lower than that of other selective and differential media. The use of the KR medium for the selective isolation of *Cronobacter* spp. in laboratories and food industry settings may therefore lessen the financial burden of *Cronobacter* spp. detection.



## 1. Introduction

*Cronobacter* spp., a novel genus comprising 6 species previously classified as *Enterobacter sakazakii* (Iversen et al., 2007), is an opportunistic pathogen associated with various foodborne illnesses mainly in neonates and infants who are easily exposed to food-borne diseases due to their weak immune system. *Cronobacter* has been shown to be the aetiological agent of life-threatening infections, causing fatal neonatal meningitis, sepsis and necrotizing enterocolitis (Bar-Oz et al., 2001; Muytjens et al., 1983; Van Acker et al., 2001). Powdered infant formula (PIF) and powdered milk have been reported as the main sources of the pathogen, which currently represents a great concern for the dairy industry (Jang and Rhee, 2009; Kim et al., 2010; Weir, 2002). *Cronobacter* spp. has been also detected in various other food sources, including bread, meat, sausage, seeds and vegetables (Gassem, 1999; Iversen and Forsythe, 2004; Robertson et al., 2002; Soriano et al., 2001), as well as in environments such as hospitals (Masaki et al., 2001), manufacturing facilities and households (Kandhai et al., 2004).

Violet red bile glucose agar (VRBGA) and tryptic soy agar (TSA) have been previously recommended by the U.S. Food and Drug Administration (FDA) for the isolation of *Cronobacter* spp. (USFDA, 2002). In the suggested protocol, enriched samples of *Enterobacteriaceae* in enrichment broth (EE broth) are plated onto VRBGA, after which presumptive colonies (purple colonies surrounded by purple halo) are streaked onto TSA and incubated at 25 °C for 48 to 72 h. Yellow pigmented colonies on TSA are confirmed with the API 20E biochemical identification system and a negative result for the oxidase test. However, reevaluation of this method has been prompted because of its overly long detection period (5–7 days) and poor level of differentiation. Recently, a new method incorporating two components, a real-time PCR and culture confirmation using Druggan Forsythe Iversen (DFI) medium and *Enterobacter sakazakii* chromogenic plating medium (ESPM), was devised by the FDA (Lampel and Chen, 2009).

Several previously introduced selective and differential media have been developed based on the report by Muytjens, van der Ros-van de Repe, and van Druten (1984) that *Cronobacter* spp. has a unique α-glucosidase activity. Among these are the DFI medium (Iversen et al., 2004), the Oh and Kang medium (OK medium) (Oh and

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Kang, 2004), ESPM (Restaino et al., 2006), and Enterobacter sakazakii isolation agar (ESIA), recommended by the International Organization for Standardization-International Dairy Federation (Besse et al., 2006). In the DFI and ESIA media, the presence of 5-Bromo-4-chloro-3-indolyl-α-D-glucopyranoside (XαGlc) serves as an indicator of α-glucosidase production, whereas the ESPM contains two chromogenic substrates (XαGlc and 5-bromo-4-chloro-3-indoxyl-β-D-cellobioside, XβCel) used to detect the activity of α-glucosidase and β-cellobiosidase, and three sugars (sorbitol, D-arabitol, and adonitol) used as differential agents. In the fluorogenic medium (OK medium), a fluorogen (4-methylumbelliferyl-α-glucoside, MUG) acts as a differential agent (Oh and Kang, 2004). When used as a substrate of α-glucosidase, MUG becomes fluorogenic by cleavage of the free 4-methylumbelliferyl moiety, generating fluorescent *Cronobacter* spp. colonies upon exposure to long-wave UV radiation. Although these selective and differential media have high sensitivity and specificity, they are generally too expensive and impose a financial burden on laboratories and food industries.

The objective of this study was to develop a new selective, differential and cost-effective medium for the detection of *Cronobacter* spp. by using salicin. Four strains of *Cronobacter* spp. and 40 strains of other organisms, mainly food borne pathogens and spoilage bacteria, were used to determine the media composition. Validation of the developed medium (Kim and Rhee medium, KR medium) was performed using salicin fermenting organisms including *Cronobacter* spp. (52 strains), *Enterobacter cloacae* (50 strains) and *Klebsiella pneumonia* (10 strains) isolated from clinical specimens and various food sources. In addition, the KR medium was verified in PIF artificially inoculated with healthy, heat-injured, and desiccation-injured cells of *Cronobacter* spp.

## 2. Materials and methods

### 2.1. Test organisms

Strains of *Cronobacter* spp. (4 ATCC strains and 52 strains isolated from clinical specimens and food sources) and 40 strains of other organisms (groups B-U, Table 1) were obtained from the Food Microbiology Culture Collection at Korea University (Seoul, Korea). Ten strains of *K. pneumonia* and 50 strains of *E. cloacae* isolated from clinical specimens were kindly provided by Korea University Medical Center (Seoul, Korea). Isolated strains (*Cronobacter* spp., *E. cloacae* and *K. pneumonia*) were identified by Vitek GNI system (Biomerieux, Marcy l'Etoile, France) before the experiments.

### 2.2. Culture and cell suspension

Each strain from laboratory stock cultures was streaked onto TSA, incubated 37 °C and maintained at 4 °C, with subculture performed at monthly intervals. *Cronobacter* spp. was enriched by growing cultures in 10 ml EE broth in screw-cap tubes at 37 °C for 24 h. Other strains were separately activated through the transference of inoculating loops to each enrichment broth as stipulated in the FDA isolation protocol and incubated accordingly. To obtain a similar cell concentration of each species, each enriched bacteria culture was diluted to $10^5$–$10^6$ CFU $ml^{-1}$. Ten-milliliter cultures of each group were combined in a plastic 50-ml centrifuge tube (Becton Dickinson, Franklin Lakes, NJ, USA) and the mixed-culture suspensions were harvested by centrifugation (Centra-CL2, IEC, Needham Heights, MA, USA) for 15 min at 2600 × *g*. After the supernatant was decanted, the pellet was washed twice in 0.2% sterile peptone water, and the final pellet was resuspended in 0.2% sterile peptone water. Each group suspension was diluted in 0.2% sterile peptone water to yield approximately $10^5$–$10^6$ CFU $ml^{-1}$, respectively. One-milliliter of cell suspension from each group was transferred to 50 ml sterile conical tubes and then thoroughly mixed, to obtain the following suspension groups (Table 1): 1) *Cronobacter* spp. cocktail, group A (4 strains of *Cronobacter* spp.); 2) other organisms' cocktail, groups B–U (40 strains of other bacteria); and 3) total organisms' cocktail, groups A–U (*Cronobacter* spp. plus other organisms, 44 strains).

**Table 1**
Bacterial strains used in the present study and morphology of colonies from bacterial strains on the Kim and Rhee medium.

| Group | Strain | Morphology on KR medium |
|---|---|---|
| A | *Cronobacter sakazakii* ATCC 12868 | Violet colony surrounded by a transparent to opalescent border |
| | *Cronobacter sakazakii* ATCC 29004 | |
| | *Cronobacter sakazakii* ATCC 29544 | |
| | *Cronobacter muytjensii* ATCC 51329 | |
| B | *Pseudomonas aeruginosa* ATCC 15692 | Colorless |
| | *Pseudomonas aeruginosa* ATCC 9027 | |
| C | *Pseudomonas putida* ATCC 21025 | |
| D | *Enterococcus faecalis* ATCC 29212 | Colorless |
| E | *Citrobacter freundii* ATCC 8090 | Colorless |
| F | *Listeria innocua* ATCC 51742 | Inhibition |
| | *Listeria innocua* ATCC 33090 | |
| G | *Listeria monocytogenes* ATCC 19111 | Inhibition |
| | *Listeria monocytogenes* ATCC 19112 | |
| | *Listeria monocytogenes* ATCC 19114 | |
| H | *Staphylococcus aureus* ATCC 27664 | Inhibition |
| | *Staphylococcus aureus* ATCC 13565 | |
| | *Staphylococcus aureus* ATCC 25923 | |
| I | *Bacillus cereus* ATCC 10876 | Inhibition |
| | *Bacillus cereus* ATCC 13061 | |
| | *Bacillus cereus* ATCC 11778 | |
| J | *Klebsiella pneumoniae* Kla | Violet colony without a transparent to opalescent border |
| | *Klebsiella pneumoniae* Revco 55 | |
| | *Klebsiella pneumoniae* ATCC 13882 | |
| K | *Eenterobacter cloacae* ATCC 7256 | Violet colony without a transparent to opalescent border |
| | *Eenterobacter cloacae* ATCC 13047 | Colorless |
| L | *Enterobacter aerogenes* ATCC 13048 | Violet colony without a transparent to opalescent border |
| | *Enterobacter aerogenes* ATCC 15038 | |
| | *Enterobacter aerogenes* ATCC 35028 | |
| M | *Salmonella typhimurium* ATCC 19585 | Colorless |
| N | *Salmonella enterica* 4509 | |
| O | *Salmonella enteritidis* ATCC 13076 | |
| P | *Hafnia alvei* ATCC 29926 | Colorless |
| | *Hafnia alvei* ATCC 29927 | |
| | *Hafnia alvei* ATCC 13337 | |
| Q | *Escherichia coli* ATCC 25922 | Colorless |
| | *Escherichia coli* WADDL 2701 | |
| | *Escherichia coli* WADDL 4083 | |
| R | *Escherichia coli* O157:H7 ATCC 43894 | Colorless |
| | *Escherichia coli* O157:H7 ATCC 43895 | |
| | *Escherichia coli* O157:H7 ATCC 35150 | |
| | *Escherichia coli* O157:H7 ATCC 43889 | |
| S | *Shigella sonnei* ATCC 9290 | Colorless |
| T | *Shigella flexneri* ATCC 9199 | |
| U | *Yersinia enterocolitica* ATCC 23715 | Colorless |

### 2.3. Heat and desiccation stress

*Cronobacter* cells were heat stressed by transferring 1 ml of the *Cronobacter* spp. mixture to 9 ml of sterile 0.2% peptone water preheated (by immersing the tubes in a heated water bath) to 55 °C and applying the heat treatment for 10 min. The heated suspensions were then immediately serially diluted in room temperature, sterile 0.2% peptone water, then surface plated onto the test media and TSA, and incubated for 24 h at 37 °C.

To subject *Cronobacter* spp. to desiccation stress, a suspension of *Cronobacter* spp. cells was sprayed vertically onto the surface of an aluminum foil (placed on a laminar flow clean bench) in which a commercial milk-based powdered infant formula (Maeil Co. Ltd., Pyungteck, Korea) had been previously spread. The samples were mixed thoroughly using a sterile spatula and then dried at ambient temperature for 24 h. The powered infant formula inoculated with *Cronobacter* spp. was stored at 21 °C for 30 days. At day 0 the population of *Cronobacter* spp. was 7.66 $\log_{10}$ CFU $g^{-1}$. The water activity of non-